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14. ABSTRACT
This study was originally designed to collect data on the natural history of inhalational anthrax in a new nonhuman primate model. An uncontrollable event created a new experimental condition which allowed us to retrospectively evaluate the power of the innate immune system to protect from an aerosol exposure of B. anthracis. Five African green monkeys (AGMs) had intravenous catheters implanted. One catheter was accidentally pulled out, leaving four AGMs with catheters and one without. All were exposed, to multiple lethal doses of B. anthracis Ames strain. Blood was collected twice daily to evaluate bacteremia. The AGM with no catheter had blood drawn from a femoral vein and became bacteremic on Day 9; succumbed to inhalational anthrax on Day 10. The other four AGMs had S. marcescens contamination in the catheter; indicated by pure colonies grown from the blood. None of these AGMs showed clinical signs of illness, had B. anthracis or a detectable level of protective antigen in the bloodstream. It appears that the presence of S. marcescens may have induced a "Coley's toxin" effect in this experiment. The innate immune response may have protected the AGMs from a lethal inhalational dose of B. anthracis spores.

15. SUBJECT TERMS
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CASE REPORT

Nosocomial infection of *Serratia marcescens* may induce a protective effect in monkeys exposed to *Bacillus anthracis*

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Summary   This study was originally designed to collect data on the natural history of inhalational anthrax in a new nonhuman primate model. An uncontrollable event created a new experimental condition which allowed us to retrospectively evaluate the power of the innate immune system to protect from an aerosol exposure of *B. anthracis*. Five African green monkeys (AGMs) had intravenous catheters implanted. One catheter was accidentally pulled out, leaving four AGMs with catheters and one without. All were exposed, to multiple lethal doses of *B. anthracis* Ames strain. Blood was collected twice daily to evaluate bacteremia. The AGM with no catheter had blood drawn from a femoral vein and became bacteremic on Day 9; succumbed to inhalational anthrax on Day 10. The other four AGMs had *S. marcescens* contamination in the catheter; indicated by pure colonies grown from the blood. None of these AGMs showed clinical signs of illness, had *B. anthracis* or a detectable level of protective antigen in the bloodstream. It appears that the presence of *S. marcescens* may have induced a ''Coley's toxin'' effect in this experiment. The innate immune response may have protected the AGMs from a lethal inhalational dose of *B. anthracis* spores.
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Bacillus anthracis is the causative agent of anthrax, a zoonotic disease, which is also of great concern as a potential agent of bioterrorism. Infection in humans occurs as cutaneous, gastrointestinal, or inhalational depending on the route of exposure. Because inhalation anthrax is the form

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most likely to occur after a bioterrorism attack, much effort has been made by the U.S. military and others to develop animal models of inhalation anthrax to test drugs and vaccines and to study the pathogenesis of the disease.

In 1841, Dr. William B. Coley, a surgeon, observed that in a patient with an inoperable sarcoma, who subsequently contracted erysipelas (caused by *Streptococcus pyogenes*), complete regression of the tumor occurred.[1] Over the next 45 years, Coley treated nearly a thousand other patients, who had a variety of malignant tumors, with a mixture of *S. pyogenes* and *Serratia marcescens*. It was the combined injection of these two bacteria that would eventually be referred to as "Coley's toxin".[2] The exact mechanisms of tumor regression induced by treatment with Coley's toxin remain unknown; however, modern research supports the concept that infection-stimulated tumor regression generally results from a non-specific innate immune response.[3]

Here, we report the survival of four naïve (unvaccinated) African green monkeys (*Chlorocebus aethiops*) (AGMs), after exposure to multiple lethal doses of aerosolized *B. anthracis*. All developed a systemic nosocomial infection with *S. marcescens*, and we present preliminary results which suggest a mechanism of action similar to that of Coley's toxin (i.e., induction of the innate immune response). Reports of unlikely survival, in patients with poor prognosis from other diseases, after 'treatment' with Coley's toxin or similar therapy, suggest that stimulation of the innate immune system could be sufficient to protect AGMs from otherwise fatal inhalation anthrax.[4–6]

The study described herein was designed to collect data on the natural history of inhalation anthrax in a new nonhuman primate model using AGMs. Five AGMs had central venous catheters (CVC) surgically implanted to facilitate frequent blood collection (approximately every 12 h from Day 0 to Day 3; daily on Day 4–12, 15, 28, and 35). All were exposed to approximately 1200 LD_{50} (lethal dose in 50% population) of *B. anthracis* (Ames strain) spores via an automated bioaerosol exposure system.[7] While the AGMs were in the exposure chamber, air samples from the chamber were collected in media and subsequently cultured so that the doses could be calculated. Therefore, we are confident that all AGMs were indeed exposed to extreme lethal doses and should have succumbed to disease; it has not been the author's experience that an untreated nonhuman primate survives such a dose.

During the aerosol exposure, the CVC from one AGM, #V509, was inadvertently removed, leaving four of five AGMs with catheters. Blood was collected to evaluate bacteremia and the presence of protective antigen (PA). It was at this point the natural history study, as designed, actually became a different experiment. We made an interesting observation in this small group of AGMs.

The AGM without a CVC had blood drawn from a femoral vein and became bacteremic (with only *B. anthracis*) on Day 9 postexposure. The animal succumbed to inhalation anthrax on Day 10. V509 had multiple gross and histologic lesions consistent with inhalational anthrax (necrotizing splenitis, necrotizing hepatitis, lymphoid depletion of tracheobronchial and mediastinal lymph nodes, pulmonary and mediastinal edema with many gram-positive bacilli). Immunohistochemistry for *B. anthracis* capsular antigen in V509 identified positive bacilli in the lungs, spleen, tracheobronchial and

mediastinal lymph nodes. The four other AGMs with CVC had *S. marcescens* contamination in the catheter that was discovered from laboratory tests designed to identify bacteremia due to *B. anthracis*. Once the contamination was identified, samples were collected from the CVC and the femoral vein to confirm the systemic infection. *S. marcescens* was identified morphologically and then confirmed by both Vitek and 16S rRNA PCR sequencing. None of these AGMs showed clinical signs of illness or had *B. anthracis* bacteremia. Using an electrochemiluminescence assay, PA (secreted by *B. anthracis*) was not detected in the blood of any of the four surviving AGMs. These tests were completed on samples obtained at the same time-points as ones for the blood culture, so the results are consistent with negative bacteremia. All four AGMs were still positive for *S. marcescens* in blood culture on Day 35 and were euthanized on Day 42 postexposure. At necropsy, complete gross and histologic examination revealed no lesions consistent with inhalational anthrax. Sections of lung, spleen, tracheobronchial lymph node, and mediastinal lymph node were negative for gram-positive bacilli using Lily-twort Gram stain. Immunohistochemistry for *B. anthracis* capsular antigen were negative on sections of lung, spleen, tracheobronchial lymph node and mediastinal lymph node. Histologic diagnoses of the four AGMs included subacute to chronic mild pleuropneumonia of unknown etiology with generalized marked lymphoid hyperplasia of many lymphoid tissues. A nonspecific change of lymphoid hyperplasia was consistently present in the AGMs with CVC and tissues included the white pulp of the spleen, multiple lymph nodes (axillary, tracheobronchial, and mesenteric), bronchial associated lymphoid tissue (BALT), and gut-associated lymphoid tissue (GALT).

Unfortunately, only a limited amount of stored serum was available for cytokine evaluation. Because cytokine analysis was not part of the original study design, a comprehensive data set is not available. However, we found in the AGM that succumbed to anthrax, concentrations of both interferon-gamma (IFN-γ) and interleukin-2 (IL-2) were below detectable levels. In the four AGMs with the subclinical infection of *S. marcescens*, which survived inhalation anthrax, concentrations of serum IFN-γ ranged from 119 to 1170 pg/ml and IL-2 from 2346 to 6951 pg/ml. This suggests that there was stimulation of the innate immune system in the AGMs infected with *S. marcescens*, but not in the one AGM that died of inhalation anthrax and was not infected with *S. marcescens* (Table 1).

We acknowledge that definitive conclusions cannot be drawn from a study with such small numbers of AGMs, but propose that the subclinical infection of *S. marcescens* protected these animals from a very high aerosol dose of *B. anthracis*. The contamination in the catheter appears to have induced an innate immune response in the host, which was significant enough to protect AGMs from a lethal aerosol dose of *B. anthracis* spores. The similar lung pathology found in the four "protected" AGMs was not seen in the one that died from inhalation anthrax. We cannot conclusively determine that these changes were the result of *B. anthracis* infection, but it is strongly suggestive that the disease was attenuated by a host response. Additional studies are needed to investigate this interesting serendipitous finding and perhaps conclusively show that a "Coley's toxin" effect can induce protection against lethal inhalation anthrax. The

Table 1 Summary of study results

ID#	B. anthracis exposure dose (# LD_{50})	Catheter	B. anthracis bacteremia	S. marcescens bacteremia	Outcome	Cytokine results (pg/ml) (Day postexposure)
V258	1200	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 3	Survived	IFN-γ = 247 (21) IL-2 = ND
V299	1100	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 0	Survived	IFN-γ = 119 (21) IL-2 = 6951 (8)
V500	1400	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 1	Survived	IFN-γ = 518 (11) IFN-γ = 1170 (35) IL-2 = 3605 (8)
V506	1800	Yes	No	Yes (Day 0) >1500 cfu/ml by Day 1	Survived	IFN-γ = 211 (11) IL-2 = 2346 (12)
V509	760	No	Yes (Day 9)	No	Died[a]	IFN-γ = ND IL-2 = <limit of detection

ND = not detected; cfu = colony forming units.
[a] Succumbed to inhalation anthrax on Day 10 postexposure.

observations reported herein suggest that therapeutic countermeasures that stimulate the host's innate immune response may protect from an otherwise fatal infectious disease.

Acknowledgment

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